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(57) Abstract

A biochemical assay, for example an immunoassay or DNA probe assay is carried out by biochemically binding to a solid support an amount of an enzyme dependent upon the amount of the target substance present in the sample, and determining the amount of enzyme bound to the solid support electrochemically by measuring the electrical change produced by a redox reaction at an electrode. The bound enzyme does not itself catalyse a redox reaction, but catalyses a reaction which results in the production of a trigger substance for a cyclic chemical reaction or series of reactions, at least one product of which is the said trigger substance and at least one further product of which is capable of causing a redox reaction to take place at the electrode, which can be determined electrochemically.

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METHOD AND APPARATUS FOR BIOCHEMICAL ASSAY

This invention relates to methods and apparatus for biochemical assay, and in particular to immunoassays and nucleic and probe assays in which at least one reagent is linked to an enzyme.

Immunoassays make use of biochemical substances which have specific reactivity with other substances, for example antigens and antibodies. Immunoassay techniques are increasingly used in medicine for the detection of a wide range of substances, for example hormones, for purposes as diverse as the diagnosis of cancer, to the detection of oestrus in cattle.

In a nucleic acid probe assay, a particular nucleic acid sequence in the DNA or RNA extracted from bacterial plan or animal cells may be detected by the use of a complimentary probe containing a known sequence of nucleotide bases.

Early biochemical assays were immunoassays based mainly on the incorporation in one of the components of the reaction of a radioisotope, or a fluorescent group as a label, so that the presence of a particular reaction product can be determined qualitatively and quantitatively.

Both of these techniques have disadvantages associated with them. Both require sensitive and expensive equipment. In addition, there are practical

difficulties in the extent to which radioisotopes can be incorporated in antigens and antibodies, and the products may have short shelf lives. The use of fluorescent groups results in an assay which is difficult to standardise because of the background fluorescence of biological material so that quantitative results cannot be obtained easily.

The use of enzymes as labels in immunoassay has been a recent development and has offered the advantages of long shelf life, safe reagents and assay end points that can be read using readily available optical equipment. Conventional enzyme immunoassay has not, until recently offered an improvement in sensitivity over radio-immunoassay.

In DNA probe assays, the DNA probe is conventionally labelled, for example, with a radioactive atom such as phosphorus, directly with an enzyme such as alkaline phosphatase or indirectly with biotin incorporated into the probe and the subsequent addition of an enzyme conjugate, for example an avidin-alkaline phosphatase conjugate. The hybridisation of the labelled probe with the target sequence in the nucleic acid can be used to immobilise an enzyme to a surface, in much the same way as in an immunoassay. This enzyme can be detected by its generation of a coloured product or, as disclosed in

International Patent Application PCT/GB84/00432 International by using an electroactive enzyme such as glucose oxidase which can be detected electrochemically.

Various techniques have recently been proposed for example in EP 37036, EP 19606, EP 58635 and EP 60123 for improving the sensitivity of enzyme assays, by the use of so-called "biochemical amplifiers".

In existing biochemical amplified systems, an enzyme-based immunoassay generates a trigger substance for a cyclic series of reactions, which can produce a much more substantial change than the conventional direct assay method. To date however, such amplified systems have been restricted to biochemical amplifiers which produce a colour change, which is measured optically.

In practice, these enzyme immunoassays are generally carried out by using a test plate having a large number of test wells, normally 96, and an optically clear bottom plate. Upon completion of the immunoassay, the test plate is inserted into a measuring instrument and an optical beam can be passed through each of the test wells in turn, so as to provide a reading of change in optical density for the various test wells. Each immunoassay is normally carried out in duplicate (ie. in at least two test

wells), and in addition, various blanks and standards are needed in order to calibrate the results obtained. Because the optical measurement on each well takes a finite time, and the plate has to be physically moved between each measurement, the scanning of the various test wells for even a single determination can take a substantial length of time, and because of the limited dynamic range of optical instruments it also makes it very difficult to obtain during the same test run accurate results for test solutions where the concentration of the test substance varies widely.

European Patent Specification No. 0150999 discloses an immunoassay method in which an enzyme label which catalyses a redox reaction, e.g. glucose oxidase, is bound to a stationary support in an immunoassay, and the products of the redox reaction which it catalyses are determined, indirectly via a ferrocene mediator, electrochemically. EP 0150999 is concerned solely with assays which bind an enzyme label which itself catalyses a redox reaction, the product of which may be determined, directly or via a mediator at an electrode surface. The sensitivity of the assays disclosed is low, and generally not sufficient to enable a practical assay to be constructed which is sufficiently sensitive to detect the low levels of analytes which are frequently

encountered.

We have now found that if an enzyme immunoassay is carried out in which the bound enzyme label is not an enzyme that can catalyse a redox reaction directly, but instead catalyses a reaction, typically a hydrolytic reaction, which results in the production of a trigger substance for a cyclic chemical reaction or series of reactions, which generates a further product capable of taking part in an electrochemical redox reaction at an electrode, an electrochemical immunoassay can be produced which is of substantially enhanced sensitivity. The term "product" used above is intended to include a product of a cyclic reaction which is continuously produced and consumed during operation of the cycle.

The cyclic chemical reaction will in general itself be a redox cycle, although it may be a non-redox cycle, one product of which is capable either of reacting further at an electrode or of producing a further product which subsequently takes part in electrochemical oxidation or reduction at an electrode.

In such redox cycles one of the products of the cycle (for example NADH, in an NAD^+/NADH cycle or quinone in a catechol/quinone cycle) is capable of being, respectively, oxidised or reduced, either at

the electrode itself, to produce a determinable electrochemical change, or via the active site of a suitable enzyme, or else in solution, to produce a change of the oxidation state of a mediator, which can then be determined electrochemically at the electrode. When the cyclic reaction is an NAD^+/NADH cycle, the NADH produced may be determined directly at a suitable electrode, for example a graphite electrode, doped with a suitable modifier, e.g. thionine, or benzoquinone. In an alternative and preferred embodiment, a soluble mediator or combination of mediators is employed, such as ferrocene, hexacyanoferrate, or phenazinium methyl sulphate (PMS).

When a chemical mediator is employed, a metal electrode may be utilised, for example a gold or platinum electrode.

Determination of the redox reaction at the electrode may be made by standard voltammetric techniques.

The immunoassay may be carried out so as to immobilise the enzyme label on any convenient surface, for example a wall of a test cell, or, as disclosed in European Patent Specification No. 0150999, on the surface of magnetic particles. Alternatively, the enzyme label may be immobilised directly on the

surface of an electrode.

It is particularly advantageous in an immunoassay to assemble together a plurality of test cells, each having electrodes connected to the tracks of a printed circuit board. This makes it possible to monitor simultaneously the progress of a large number of enzyme reactions, representing separate immunoassays, at relatively short time intervals, for example using a computer.

Accordingly, in a second aspect of the invention, there is provided a biochemical test cell assembly comprising at least one test cell for containing a test solution, a pair of electrodes in the test cell, and a biochemical ligand bound to a surface in the cell, the said ligand being adapted to bind specifically with a corresponding antiligand, thereby to immobilise on the said surface an enzyme label capable of being detected, directly or indirectly, by means of its electrical effect on the said electrodes, and preferably by its ability to trigger a further chemical reaction employing a redox cycle with an electrical effect on the said electrodes.

As indicated above, the assembly preferably includes a plurality, for example at least eight, test cells, each being connected to a track of a printed circuit board, which forms a part of the assembly.

The printed circuit board preferably also incorporates a multi-way connector, to enable the simultaneous connection of measuring equipment to the various pairs of electrodes in the assembly. The measuring equipment may take the form of a micro-computer, including a micro-processor, which is adapted to read repeatedly a sequence of values from the electrode pairs, for example using a single analogue-to-digital converter and sequencing switch, and store the values associated with each reading, so as to provide a profile of the change in the said electrical value, for each of the electrode pairs, over a period of time.

The surface to which the ligand is bound may be one of the electrode surfaces, so that the antiligand to which the enzyme label is attached is conveniently situated close to the respective electrode, in order to facilitate electron transfer between the electrode surface and the substance which takes part in the electrochemical redox reaction.

When a biochemical amplifier is used (i.e. when the enzyme label catalyses a reaction which results in the production of a trigger substance for a cyclic chemical reaction, as described above), the ligand need not necessarily be bound to the electrode surface as the enzyme label may be measured indirectly by its

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triggering effect on the components of the biochemical "amplifier" which may be freely diffusing or some or all of which may be immobilised to the electrode surface to enhance electron transfer.

In a further embodiment of the invention, one electrode of the or each pair may be attached to a removable cover portion for the test cell. Thus, in an alternative aspect of the invention, there is provided a cover for a test cell assembly, comprising a plurality of pairs of electrodes mounted on and projecting from the cover at least one of the electrodes of each pair preferably having a biochemical ligand bound to its surface.

The method of the invention is equally suited for use in DNA probe assays. In a DNA probe assay in accordance with the invention the label which is utilised is the said enzyme, for example a phosphatase or a hydrolase, which does not itself catalyse a redox reaction, but catalyses a reaction which produces a trigger substance as indicated above. It is particularly advantageous in this case for the DNA or RNA hybridisation assay to be carried out in a nitrocellulose or nylon membrane or filter which is in direct contact with a pair of flat platinum electrodes bonded to the lower surface of the matrix. In this way the activity of the biochemical amplifier can be

detected by the simple addition of the appropriate reagents to the filter and the application of a suitable potential to the electrodes.

A number of the particular embodiments of the invention will now be described, and are illustrated with reference to the accompanying drawings, in which:-

Figure 1 is a schematic representation of a reaction scheme, in accordance with the invention,

Figure 2a to 2e are schematic representations of alternative reaction schemes,

Figure 3 is a schematic representation of the operation of the lipoamide dehydrogenase active site in a redox cycle involving NADH and an electrode,

Figure 4 is a schematic diagram of a test cell assembly according to the invention,

Figure 5 is a schematic diagram of a computer system adapted for use with the test cell assembly of Figure 4, and

Figures 6, 7 and 8 are experimental plots obtained in immunoassays.

In the embodiment of the invention illustrated schematically in Figure 1, a surface 1 within a test cell which may be for example the wall of the test cell, a magnetic particle within the test cell, or the surface of an electrode, is prepared by binding an

antibody 2, preferably a monoclonal antibody, to its surface. An antigen 3 to be determined is prepared in appropriate concentration, and caused to react selectively with the antibody. A second monoclonal antibody 4, having an enzyme 5, for example alkaline phosphatase, conjugated thereto is there caused to react with the antigen 3, according to the well known "sandwich" immunoassay technique.

The method of the invention is equally applicable to non-sandwich type assays, for example to so-called "competition" assays in which the antigen competes with an antigen-enzyme conjugate for immobilised antibody. Competition assays are particularly suitable for assaying small antigens and haptens. The electrochemical detection of the bound enzyme label is the same whatever the specific mechansims of the assay.

The amount of alkaline phosphatase linked to the surface 1 is proportional to the amount of antigen 3 in the sample, and can be determined electrically, as follows. The support, with bound alkaline phosphatase, is washed, and a solution is added containing nicotinamide adenine dinucleotide phosphate ($NADP^+$).

The $NADP^+$ provides a substrate for the alkaline phosphatase, which catalyses the production of

nicotinamide adenine dinucleotide (NAD^+) by hydrolysis of a phosphate ester chemical bond. This NAD^+ serves as a trigger substance for a cyclic reaction (a so-called "biochemical amplifier"), resulting in the oxidation of a substrate in excess such as ethanol to acetaldehyde and the reduction of NAD^+ to NADH, catalysed by an NAD^+ specific dehydrogenase such as alcohol dehydrogenase. The NADH so formed is reoxidised by the active site of the enzyme diaphorase which is itself subsequently oxidised electrolytically at the electrode surface. This results in a measurable current, the magnitude of which is dependent upon the amount of NAD^+ produced by the enzyme label in the immunoassay. The great specificity of the enzymes alcohol dehydrogenase and diaphorase means that the substrate for alkaline phosphatase, $NADP^+$, is unlikely to enter the redox cycle.

In the example given in Figure 1 the electrode will accept electrons directly from the active site of the enzyme diaphorase. In an alternative embodiment the electrode in suitably modified form will accept electrons from NADH without the use of the enzyme diaphorase.

In a system such as this, it is not essential that the antibody 2 should be bound directly to the

electrode surface, and the surface 1 can represent any suitable surface within the test solution, for example a wall of the test cell.

Figure 3 illustrates in more detail the reaction scheme for a redox cycle involving NADH, the active site of diaphorase (known systematically as lipoamide dehydrogenase) shown schematically as a disulphide bridge and a molecule of FAD, and an electrode surface. NADH first binds to one of the sulphur atoms as shown in step one of Figure 3, to form 2 free radicals. NAD^+ then departs from the active site (step 2), and a rearrangement occurs (step 3) to reoxidise the FAD and reduce the remaining sulphur. The active site is then fully reoxidised by contact with an electrode or mediator in step 4 in which the electrons are donated to the electrode or mediator, and the disulphide bridge is reformed.

The Eo^1 value for the $NADH/NAD^+$ redox system is -320mV while that for the $FADH^2/FAD$ system in the lipoamide dehydrogénase active site is close to 0mV. Electron acceptors such as ferricyanide ($E0^1$= +418mV) or ferrocene derivatives ($E0^1$= +300 to +650mV) or an electrode at a suitable potential will re-oxidise the diaphorase active site.

Figure 2a illustrates a similar reaction scheme to that of Figure 1, but in which the NADH produced in

a cyclic reaction is used to reduce a mediator, the ferricyanide ion, to ferrocyanide, a process catalysed by the active site of diaphorase. The ferrocyanide in turn is oxidised by a platinum working electrode at a potential of +450mV, to produce a current. Because the amount of ferrocyanide produced is dependent upon the amount of NAD^+ introduced into the cyclic reaction of the biochemical amplifier, which is in turn dependent upon the amount of alkaline phosphatase 5 bound to surface 1, the steady state current produced at the electrode is dependent directly upon the amount of antigen 3 bound in the immunoassay.

A further alternative scheme is illustrated in Figure 2b, in which an additional mediator, ferrocene is interposed between the NAD^+/NADH cycle, and the ferricyanide/ferrocyanide cycle. In this case, NADH produced by the cyclic reaction of NAD^+ reduces ferricene to ferrocene, catalysed by diaphorase, which in turn reduces ferricyanide to ferrocyanide. Again, ferrocyanide is oxidised at a platinum working electrode at a potential of +450mV, to produce a current which is measured.

There are a number of alternative chemical means to produce NAD^+ from precursors that are inactive in the biochemical redox amplification cycles described

above. One alternative to the alkaline phosphatase reaction employs NAD^+ glycohydrolase as the enzyme label. This enzyme will produce NAD^+ from an analogue, NAD^+ dihydroxyacetone by a transfer reaction driven by an excess of nicotinamide.

Figures 2c and 2d illustrate alternative reaction schemes. In the example of Figure 2c, bound alkaline phosphatase 5 is used to hydrolyse a thiophosphate ester, to generate a free thiol (RSH). The free thiol serves as the trigger substance for a cyclic reaction, in which the thiol is converted to the disulphide RSSR and back again. This reaction can be caused to take place under the influence of an excess of NAD^+, catalysed by a suitable enzyme. When the thiol is lipoic acid, the enzyme may be diaphorase, or when the thiol is glutathione, the enzyme may be glutathione reductase.

In Figure 2c the thiol is regenerated by electrochemical reduction of the disulphide compound at a platinum working electrode (a cathode) at a suitable potential depending on the thiol compound chosen, but without effect on the NAD^+/NADH system ($E0^1$ -320mV). An alternative redox cycle employing electrochemical oxidation of the thiol compound is shown in Figure 2d. Here the platinum working electrode (an anode) will accept electrons from R-SH,

the disulphide is reduced by an excess of NADH via diphorase or glutathione reductase.

In the reaction scheme illustrated as Figure 2e the enzyme label in the immunoassay is β-galactosidase. The enzyme catalyses the hydrolysis of p-hydroxy-phenyl β-galactoside generating 1,4 benzoquinol or catechol. This is the trigger molecule for a biochemical amplifier employing the enzyme laccase. Catechol is oxidised to 1,4 benzoquinone at the expense of oxygen, a platinum working electrode (a cathode) is used to reduce the quinone to quinol ($E0^1$= +0.28V) and the current is measured.

Varisous alternative redox cycles are possible in the method of the invention. FAD (flavin adenine dinucleotide) is a component of the diaphorase active site (Figure 3). It is also used by many redox enzymes. In the case of glucose oxidase the apoenzyme can be prepared which is electrochemically inactive. The generation of FAD, by the enzyme label in an immunoassay from a precursor such as a galactoside results in the activation of glucose oxidase which then operates as a biochemical amplifier with glucose in excess (where the redox cycle is FAD/$FADH_2$ in the active site of glucose oxidase). European Patent Specification No. 0150999 discloses a method for measuring electrochemically the activity of glucose

oxidase using the soluble mediator ferrocene monocarboxylic acid and a graphite electrode. In a further example the co-factor PQQ (pyrrolo-quinoline quinone) is used by certain redox enzymes such as methanol dehydrogenase. The generation of PQQ from a precursor such as a phosphate by the enzyme label in the immunoassay results in the activation of methanol dehydrogenase and the redox cycle $PQQ/PQQH_2$ driven by an excess of methanol can reduce an appropriate electrode using a suitable mediator.

Figure 4 is a schematic diagram of a test cell assembly according to the invention. The assembly comprises a polystyrene block 31, approximately 10mm thick, having 8 through holes 32 therein. The block 31 is secured by means of an epoxy adhesive to a printed circuit board 33. The printed circuit board 33 carries eight pairs of electrodes, 34a, 35a, 36a, etc. and 34b, 35b, 36b, etc. bonded to its surface. The electrodes 34a, 35a, 36a etc. are joined by a common copper track 40 to a terminal 41, forming a common, or ground terminal. The electrodes 34b, 35b, 36b etc. are each joined by a respective copper track 37, 38, 39, etc. to a respective terminal 42, 43, 44 etc.

Thus, the terminals 41 to 49 constitute a conventional "edge" connector as used in conventional

circuit board technology. The copper tracks 39 to 40 may preferably be formed on the underside of the printed circuit board 33, so as not to interfere with the glueing of the block 31 to the circuit board 33. A portion 52 of the block 31 is cut away so as to facilitate the use of the edge of the circuit board 33 as an edge connector. Thus, when the block 31 is glued in position on the plate 33, a test cell assembly is formed comprising a plurality of test cells defined by the holes 32, and the bottom plate 33, each having within it a pair of electrodes, which can be accessed via the edge connector constituted by terminals 41 to 49.

The 8 x 1 array of test cells illustrated is simply illustrative, and in a practical embodiment, an array of, perhaps, 12 x 8 might be used, to provide 96 test cells, each having two electrodes therein.

The test cell surfaces may be coated with a ligand material, for example a monoclonal antibody, by any known means, for example by simply introducing an appropriate solution of the antibody into the test cell, allowing it to stand, and decanting the liquid.

Figure 5 illustrates a simplified computer arrangement for carrying out an electrochemical immunoassay using the test cell assembly of Figure 4. A 9-way connector 75 adapted to connect with the

terminals 41 to 49 of the assembly Figure 4 is connected via a 9-way ribbon cable 74 to a software-driven sequence switch 70. The sequencing switch 70 connects each of the electrodes 34b, 35b, 36b etc. in turn to the input of an analogue-to-digital converter 71, in turn connected to a micro computer 73. The micro computer 73 is adapted to sample each of the cells 32 at regular intervals, for example every second, and to store the current values associated therewith. Once stored the values can be accessed as desired to provide kinetic measurements of the progress of the enzyme assays.

In an alternative embodiment, the electrodes may be formed by a plurality of pins formed on a printed circuit board, and adapted to project downwardly into the wells of a conventional optical microtitre plate, for example a plate such as that produced by NUNC. Such a board may be provided with an edge connector, as described above.

The electrode may be formed of a metal, or metal-treated plastic, carbon, or other conducting or semi-conducting material. In particular, for the direct oxidation of NADH, a suitable electrode may be produced by treating graphite with benzoquinone, or, much more preferably, thionine.

A number of particular embodiments of the

invention are described in the following examples.

Example 1

An electrochemical immunoassay for human prostatic acid phosphatase was carried out in accordance with the following method, using the principle outlined in Figure 2a.

The wells of a 96-well polystyrene plate (NUNC Immunoplate I) were coated with a monoclonal antibody to human PAP (prostatic acid phosphatase) by diluting the antibody to a concentration of 5µg/ml in 1.0mM-sodium carbonate buffer pH 9.0 and pipetting 100ul of the solution into each well. The plate was incubated for 16 hours at 37 °C and excess antibody was removed by washing with the carbonate buffer. Then, to each well was added 75µl of a solution containing 50ng/ml of a conjugate of a second monoclonal antibody and alkaline phosphatase (made according to methods given in "Enzyme Immunoassay" Ishikawa, Kawai and Miyai 1981, Igaku-Shoin, Tokyo) in 100mM-triethanolamine buffer pH 7.5 containing 6% (w/v) bovine serum albumin, 1mM-magnesium chloride, 0.05% (v/v) Triton X705 and 0.1% (w/v) sodium azide, followed immediately by 25µl of human serum containing known amounts of human PAP. The plate was incubated for 2 hours at 22 °C during which time a sandwich of immobilised antibody, PAP and conjugate formed on the

surface of the well. Excess conjugate and serum was removed by washing the wells with a solution of 0.1% Triton-X705, 25mM-Tris buffer pH 8.0 and 200mM ammonium sulphate. To each well was added 200µl of a 0.2mM solution of $NADP^+$ (nicotinamide adenine dinucleotide phosphate) in 50mM -diethanolamine buffer pH 9.5. After 20 minutes at 22 °C the enzyme reaction was stopped by the addition of 20µl of 0.10 M-sodium phosphate pH 7.0. A total of 12 replicate wells at each PAP concentration were pooled in order to provide sufficient material for the electrochemical cell.

The concentration of NAD^+ formed by the action of alkaline phosphatase on $NADP^+$ was then measured electrochemically. The cell had a volume of 2ml and contained platinum working (3mm x 0.5mm wire), and counter (3cm x 0.5mm) electrodes and a standard silver/silver chloride reference in a second compartment connected to the working cell by a capillary. A magnetic stirring bar was used to stir the contents of the cell. Additional apparatus comprised a potentiostat (Thomson Electrochem, Newcastle-Upon-Tyne) and the current flowing in the cell was monitored with a resistance box (100,000 ohms) and a chart recorder (JJ Instruments, Southampton). Prior to use the working electrode was

pretreated by applying a potential sweep from -600mV to +600mV for 10 minutes at a sweep rate of 1volt/second. (After each measurement the electrode was rinsed with distilled water, polished with an alumina (0.3μm) slurry and then rinsed again). To the cell was added 1.0ml of a biochemical "amplifier" mixture containing 0.2mg alcohol dehydrogenase, 0.2 mg pig heart diaphorase, 10% (v/v) ethanediol, 85mM-sodium chloride, 3% (v/v) ethanol in a 25mM-phosphate buffer pH 7.0, followed by 1.0ml of the pooled $NADP^+$ solutions (containing varying amounts of NAD^+) from the immunoassay. The current in the cell was measured after the addition of the ferricyanide to a final concentration of 25mM and the application of a potential of +450mV. The current increased linearly for the first five minutes of measurement and reached a steady value after 20 minutes. Both the rate of increase and the steady state value were proportional to the concentration of NAD^+ in the sample. The rate of increase of current in μA/min was plotted against the concentration of PAP in the human serum sample and the results are shown in Figure 6. The least detectable concentration of PAP in the electrochemical immunoassay, defined as 2.5 times the standard deviation on the zero standard, was 0.24 ng/ml or 60 attomoles of antigen.

It will of course be appreciated that the nature of the antigen 3 which is determined by an electrochemical immunoassay in accordance with the present invention is irrelevant, and any desired antigen that is sufficiently large to contain several epitopes can be determined, by appropriate choice of the antibody 2 bound to the surface 1, and the antibody 4, conjugated to the enzyme label 5. In the case of a small antigen, such as progesterone, a competition reaction between antigen and enzyme labelled antigen would result in an amount of enzyme label 5 being bound by antibody 2 that is inversely proportional to the amount of antigen present. In this case the electrochemical signal would reduce as antigen concentration increases.

Example 2

A thionine-modified graphite electrode was prepared as follows. 10 mg of thionine was dissolved in 2 ml of 90% ethanol. A 3 mm disc of graphite was immersed in the solution, and left for 10 minutes. The disc was removed, and washed thoroughly with distilled water. The disc was then washed in acetone, and finally sonicated in distilled water for 5 minutes. The graphite disc was attached to a platinum wire using a silver-loaded epoxy resin.

Electrical measurements were performed using a

two-compartment glass cell that had a working volume of 2 ml. In addition to the working graphite/thionine electrode, the cell contained a 3 cm long platinum wire (0.5 mm diameter) as a counter and a saturated calomel electrode as reference. The working compartment of the cell could be stirred during operation with a magnetic stirrer bar and additions could be made directly to the cell via an injection port. Cyclic voltammograms showed a complex picture with several peaks but it was found that, at a potential of +150mV, a satisfactory current could be obtained from a solution containing NADH. A series of measurements at this potential demonstrated that the response of the electrode was rapid and linear up to a concentration of 100μm - NADH in a 0.1M -sodium phosphate buffer at pH 7.0. The steady state current was reached practically instantaneously following injection of NADH and remained stable for periods in excess of 10 minutes.

An immunoassay for PAP was then carried out in accordance with the general methods described in Example 1 using the principle described in Figure 1. The concentration of NAD^+ formed at each concentration formed at each concentration of PAP was measured with the thionine-modified graphite electrode. To the cell was added 1 ml of a mixture

essentially the same as the biochemical amplifier described in Example 1 except that diaphorase and ferricyanide were omitted. Then, 1 ml of each pooled $NADP^+$ solution from the immunoassay was added and the steady state current was measured. The cell was washed with distilled water between measurements. The steady state current in the cell was plotted versus the concentration of PAP in the human serum sample and the results are shown in Figure 7. The least detectable concentration was 1.98 ng/ml.

Example 3

An electrochemical immunoassay for human prostatic acid phosphatase in a biochemical test cell assembly was carried out as follows, using the reaction scheme illustrated in Figure 2a. The wells of an 8 well polystyrene strip (NUNC) were coated with a monoclonal antibody to human PAP according to the procedures described in Example 1. The immunoassay was carried out as described in Example 1 up to the addition of 100µl of the $NADP^+$ solution to the wells. After 20 minutes of incubation (at 22°) with this substrate, 100µl of a mixture which was essentially the same as the biochemical amplifier described in Example 1, but which also contained ferricyanide at 25mM was added to each well. The phosphate buffer in the biochemical amplifier

inhibited the further reaction of the enzyme label, alkaline phosphatase, and the NAD^+ formed in each well was able to trigger the biochemical amplifier. Immediately after addition of the biochemical amplifier to each well a lid incorporating a pair for each well, of platinum working and counter electrodes (each 3mm x 0.5mm) was placed over the 8 well strip. The platinum electrodes were fully immersed in the solution in each well and were cemented to insulated copper pins 2mm in diameter using silver-loaded epoxy resin. The copper pins were pushed through holes drilled in a polystyrene sheet of dimensions 8cm x 1cm x 3mm to form the lid and each pin was connected to a software driven switch as shown in Figure 5. Prior to use the platinum working electrodes were pretreated by a potential excursion from -600mV to +600mV in distilled water and each working electrode had been polished with an alumina slurry (0.3μm). The test well assembly and lid was then taped to the top plate of a Titertek plate shaker (Flow Laboratories) and vigorously agitated to simulate a stirred cell. A potential of +450mV was applied to each working electrode in turn via the user port of a BBC microcomputer (with appropriate software for poising the potential and recording current from two electrode systems). The rate of increase of the current in each

well was measured for two minutes by the microcomputer. The rate of increase in the current (nA/min) was plotted against the concentration of PAP in the human serum sample and the results are shown in Figure 8.

In this example the activity of the enzyme label in the immunoassay was stopped before the addition of the biochemical amplifier. The alternative of allowing the activity to continue was not used owing to the more complex kinetics of current generation caused by the continuous appearance of the trigger molecule NAD^+ throughout the measurement period.

CLAIMS

1. A method of determining a biochemical substance in a sample, which method comprises carrying out an assay so as to biochemically bind to a solid support an amount of an enzyme dependent upon the amount of the said substance present in the sample, and determining the amount of enzyme bound to the solid support electrochemically by measuring the electrical change produced by a redox reaction at an electrode, characterised in that the bound enzyme does not itself catalyse a redox reaction, but catalyses a reaction which results in the production of a trigger substance for a cyclic chemical reaction or series of reactions, at least one product of which is the said trigger substance and at least one further product of which is capable of causing a redox reaction to take place at the electrode, which can be determined electrochemically.

2. A method as claimed in Claim 1, wherein the trigger substance is á nicotinamide adenine dinucleotide co- enzyme.

3. A method as claimed in Claim 1 or Claim 2, wherein the said further product is lipoamide dehydrogenase.

4. A method as claimed in Claim 1 or Claim 2, wherein one of the cyclic series of reactions is a

dehydrogenation reaction which is conducted in the presence of an NAD^+-specific dehydrogenase.

5. A method as claimed in any one of the preceding claims wherein a biochemical ligand is bound to a surface of the electrode, and the ligand reacts with a corresponding antiligand during the immunoassay.

6. A method as claimed in any one of the preceding claims, wherein the said enzyme is a phosphatase or a hydrolase.

7. A method as claimed in any one of the preceding claims, wherein the cyclic chemical reaction is an NAD^+/NADH cycle, a catechol/quinone cycle, a thiol/disulphide cycle, an FAD/FADH2 cycle or a PGG/PQQH2 cycle.

8. A method as claimed in Claim 6 and Claim 7, wherein the enzyme is alkaline phosphatase, the cycle chemical reaction is an NAD^+/NADH cycle, and the trigger substance is NAD^+.

9. A method as claimed in any one of the preceding claims, wherein the reaction mixture comprises at least one electron transfer mediator which can transfer electrons between the said further product and the electrode.

10. A method as claimed in Claim 9, wherein the electron transfer mediator is ferrocene or a derivative thereof and/or ferricyanide.

11. A biochemical test cell assembly, comprising at least one test cell for containing a test solution, a pair of electrodes in the test cell, and a biochemical ligand bound to a surface in the cell, the said ligand being adapted to bind specifically with a corresponding antiligand, thereby to immobilise on the said surface an enzyme capable of being detected by means of its electrical effect on the said electrodes.

12. An assembly as claimed in Claim 11, which comprises a plurality of test cells each containing a pair of electrodes, the electrodes of the test cells each being connected to a track of a printed circuit board forming a part of the assembly.

13. An assembly as claimed in Claim 12, including a multi-way connector to enable the simultaneous connection of measuring equipment to a plurality of pairs of electrodes in the assembly.

14. An assembly as claimed in any one of Claims 11 to 13, including means to read sequently electrical values associated with a plurality of electrode pairs.

15. An assembly as claimed in Claim 14, wherein the means for reading comprises a computer adapted to read repeatedly a sequence of values from the said plurality of electrode pairs, and store the values associated with each reading, so as to provide a

profile of the change in the said electrical value for each of the electrode pairs, over a period of time.

16. An assembly as claimed in any one of claims 13 to 15, wherein the enzyme is capable of being detected by means of its triggering effect on a cyclic reaction or series of reactions which have an electrical effect on the said electrodes.

17. An assembly as claimed in any one of claims 13 to 16, which comprises at least eight test cells, each having a pair of electrodes.

18. An assembly as claimed in any one of claims 12 to 17, wherein at least one electrode of the or each pair is attached to a removable cover portion for the respective test cell.

19. An assembly as claimed in any one of claims 12 to 18, wherein the said surface to which the ligand is bound is a surface of one of the said electrodes.

20. An assembly as claimed in any one of claims 12 to 19, wherein one of the electrode surfaces incorporates a component of a biochemical amplification cycle which cycle contains a redox reaction.

FIG. 1.

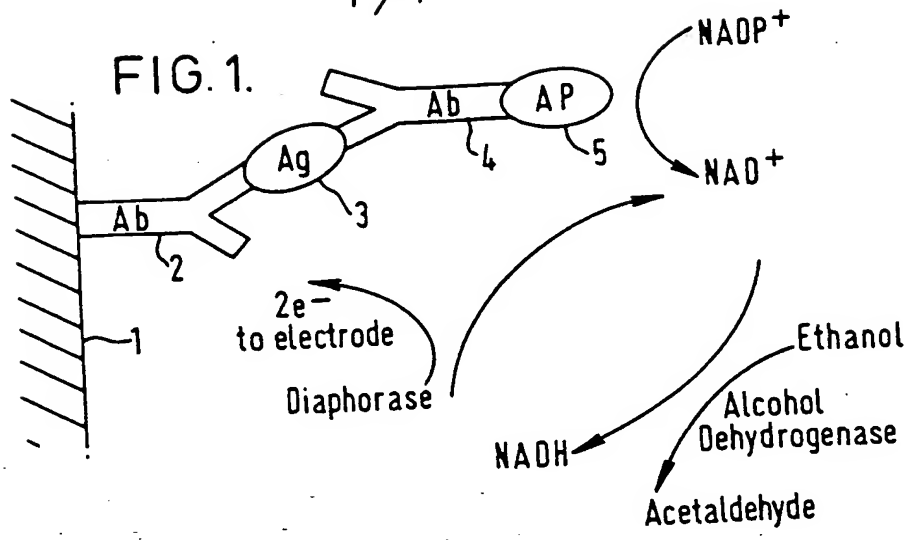


FIG. 2a.

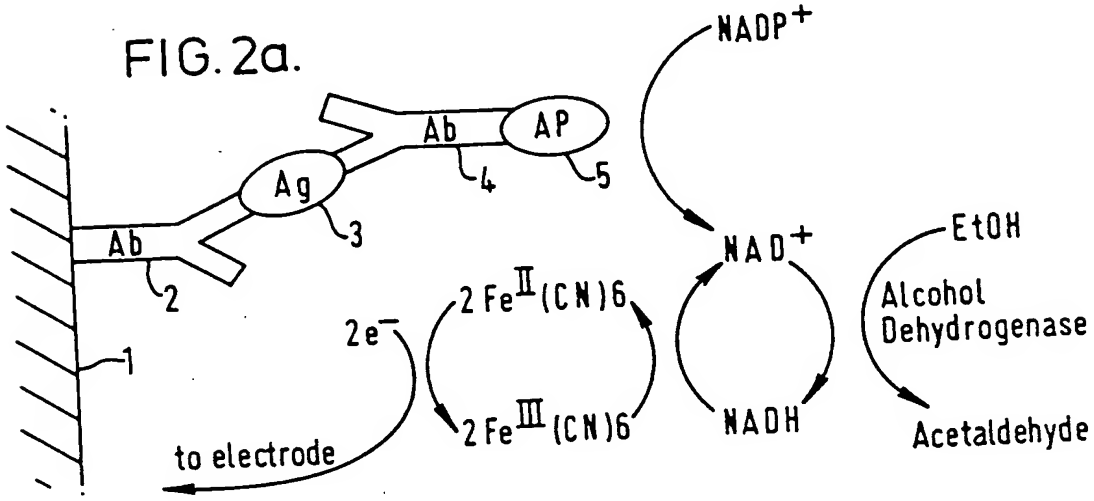


FIG. 2b.

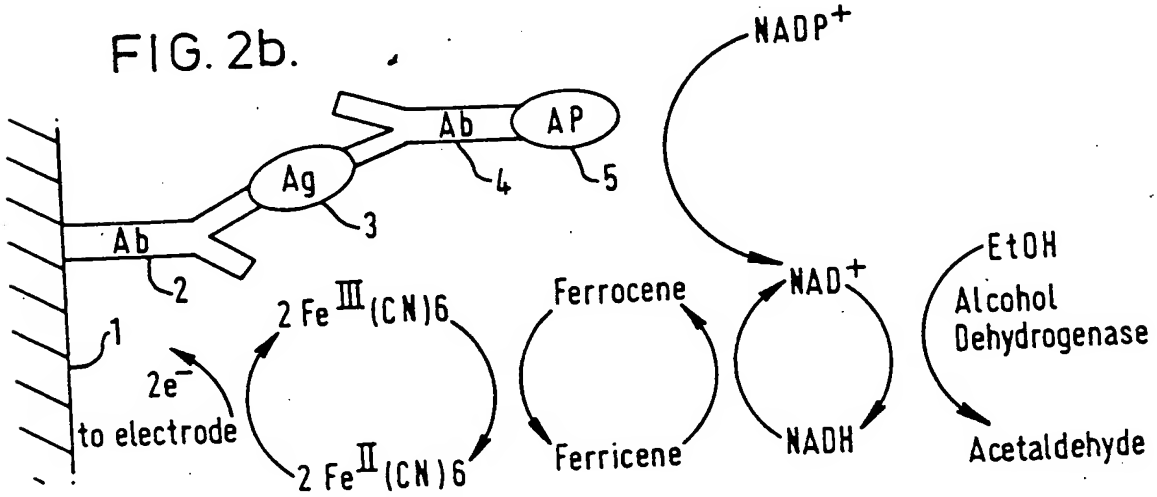


FIG. 2c.

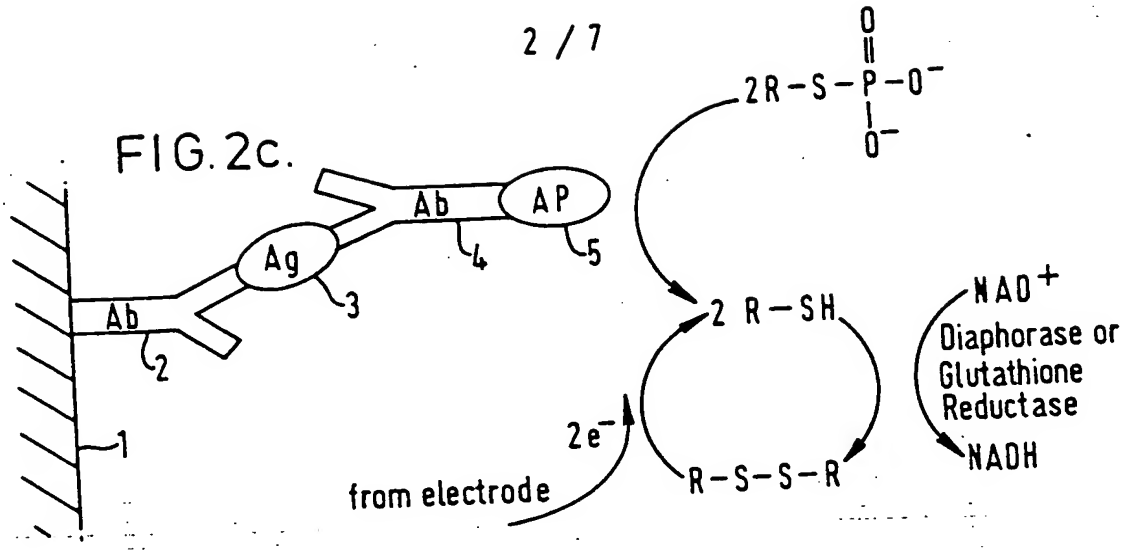


FIG. 2d.

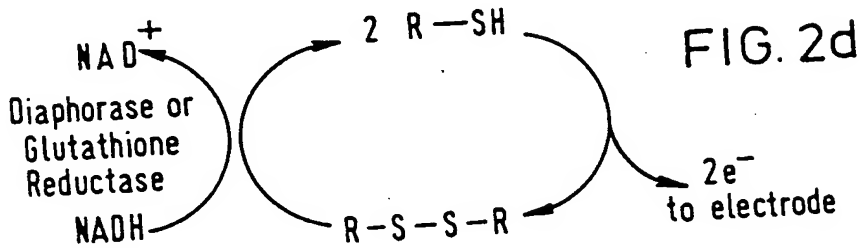


FIG. 2e.

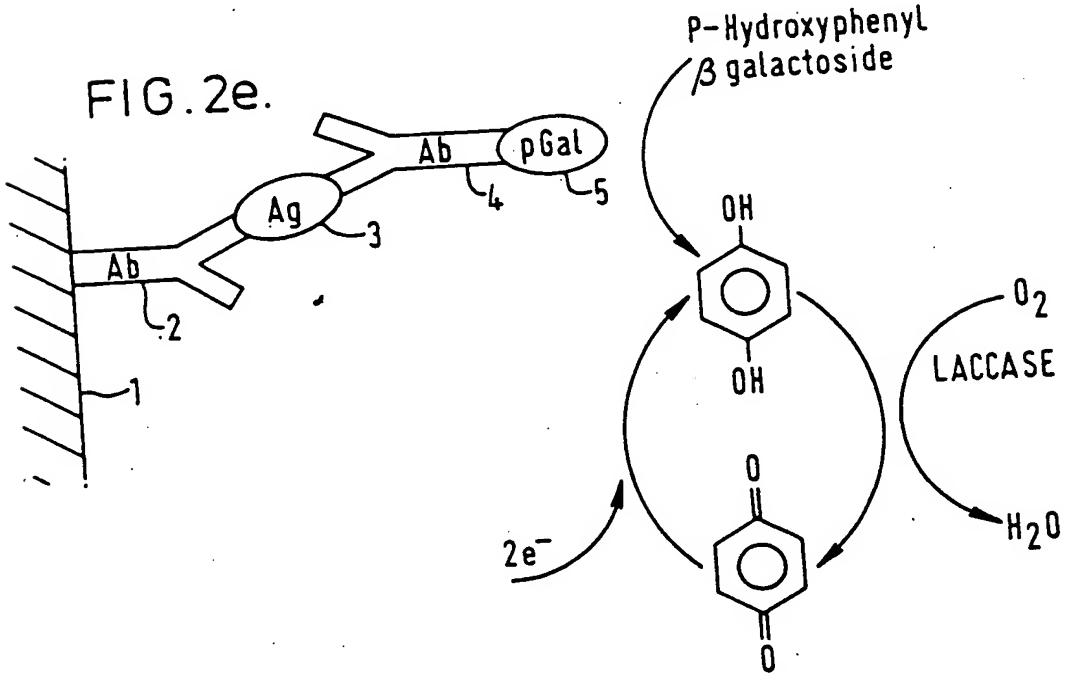


FIG. 3.

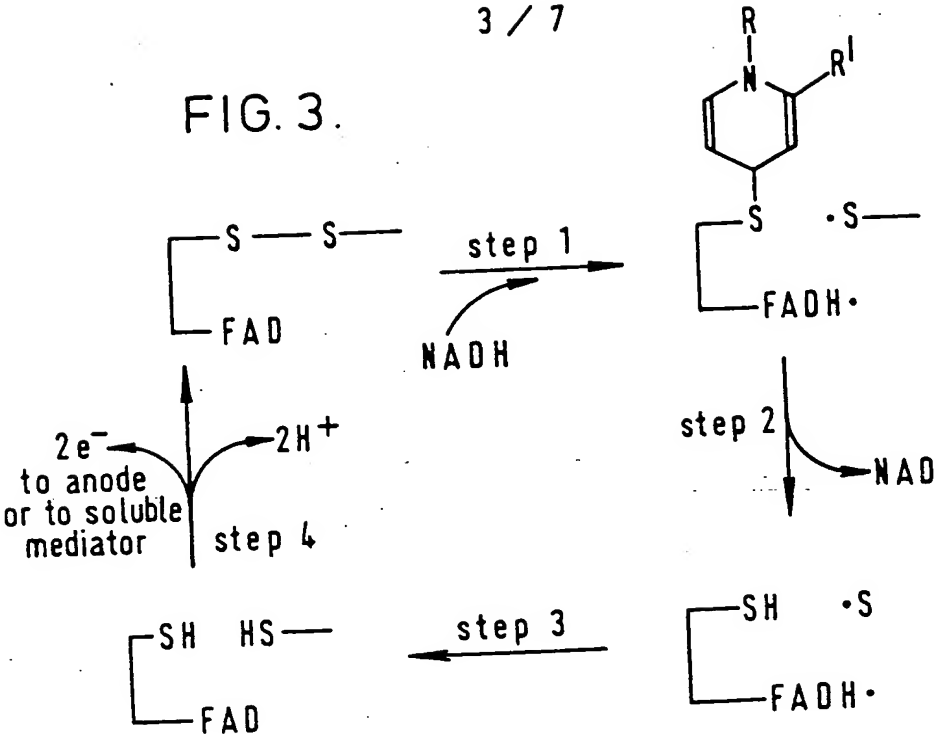
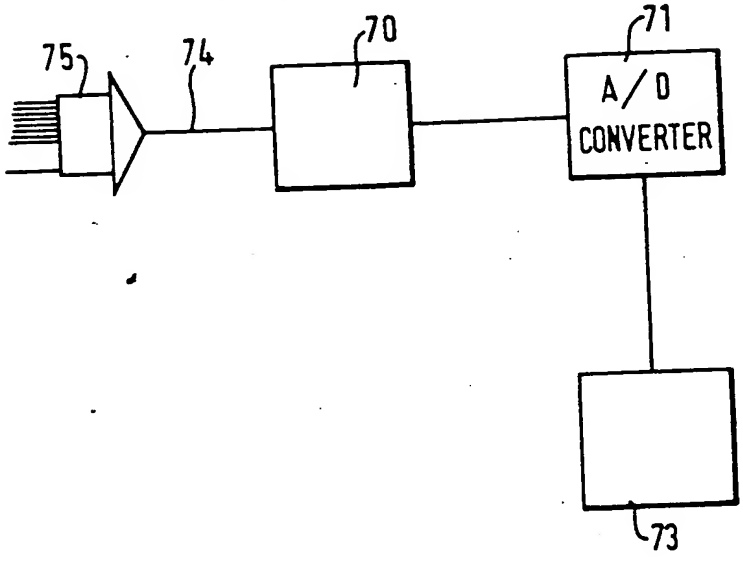

R
N
R'
S — S
FAD
step 1
NADH
S ·S
FADH·
2e⁻
to anode
or to soluble
mediator
2H⁺
step 4
step 2
NAD
SH HS
FAD
step 3
SH ·S
FADH·

FIG. 5.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A/D
CONVERTER
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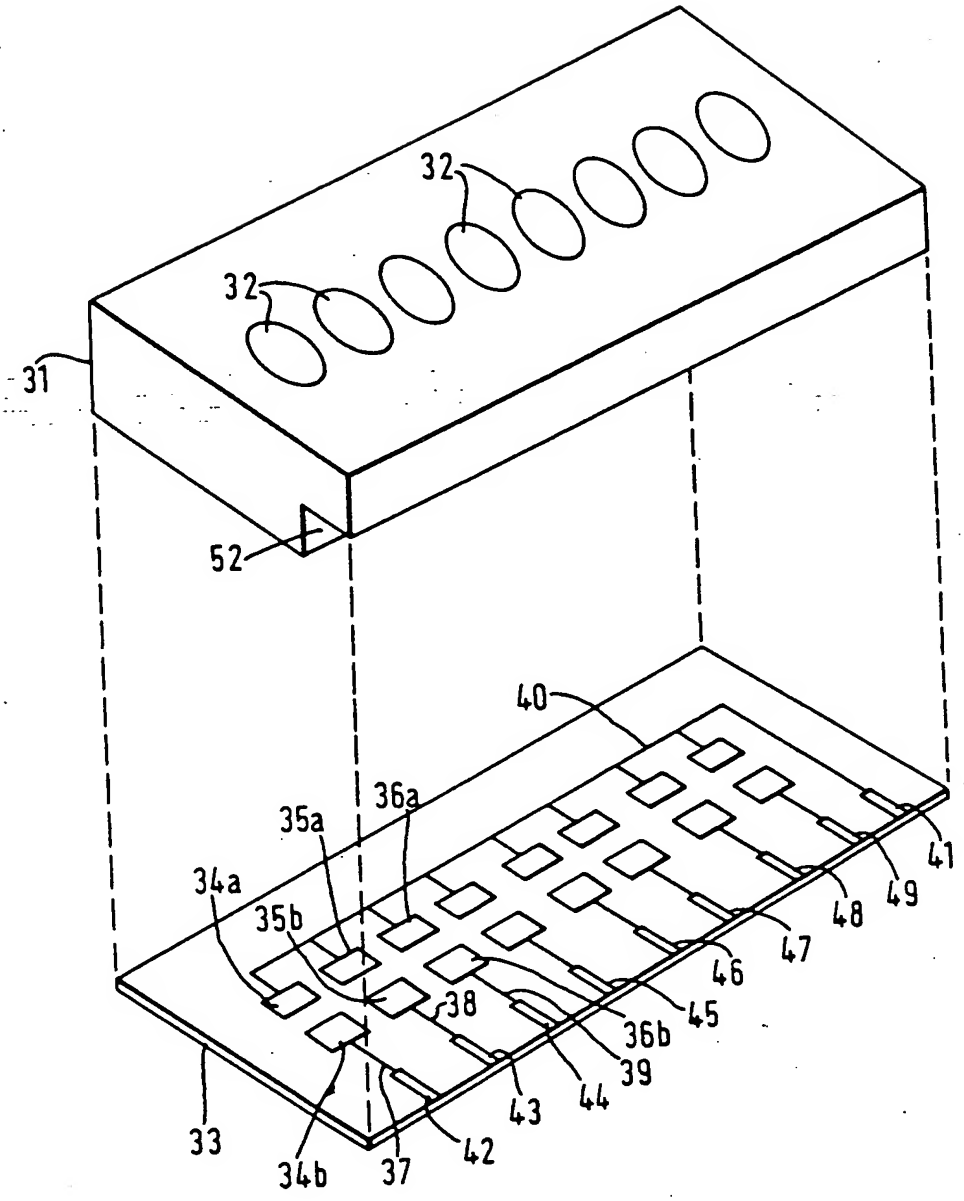
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44
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42
37
34b
33

FIG. 4.

FIG. 6.

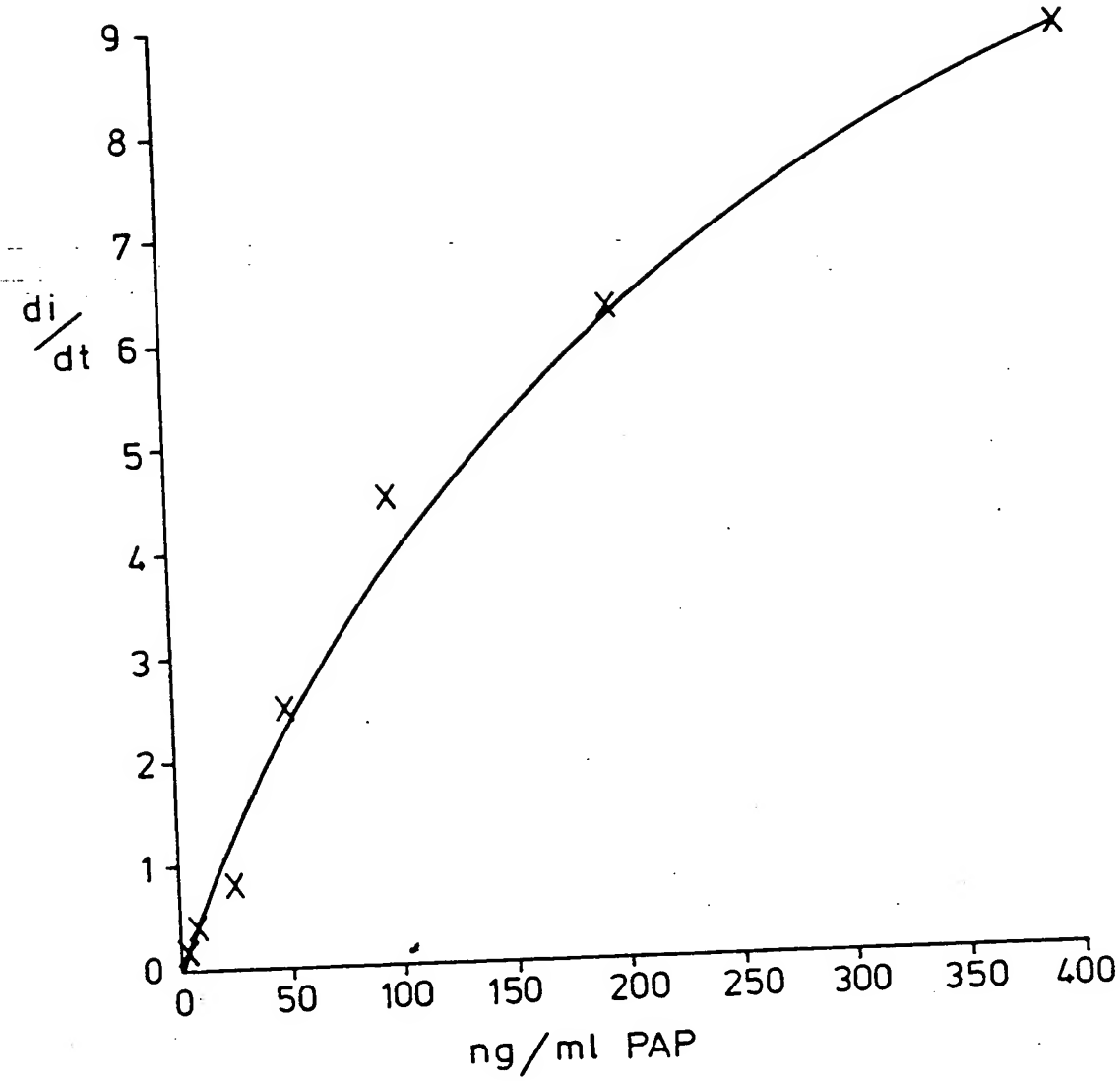
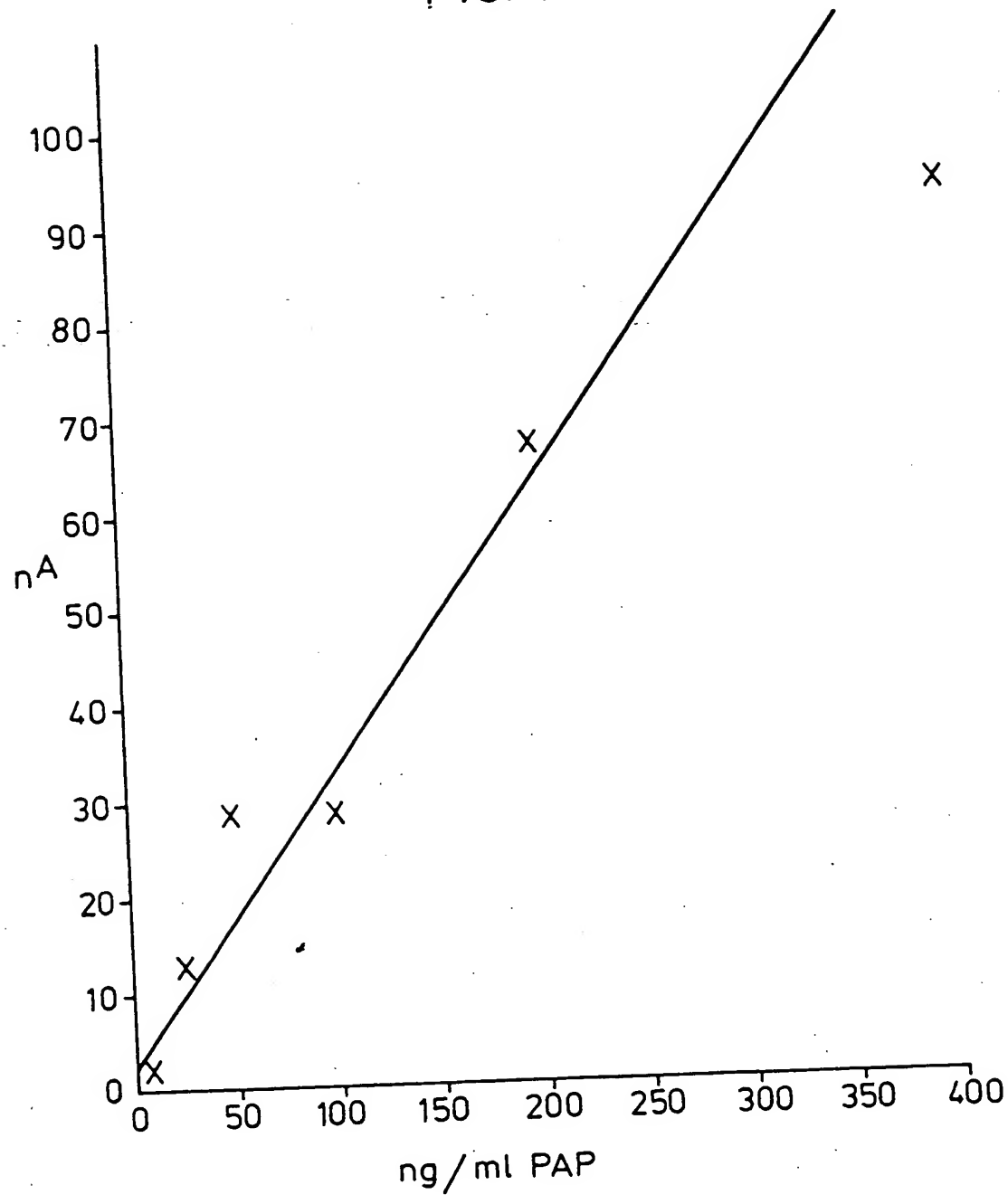


FIG. 7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350
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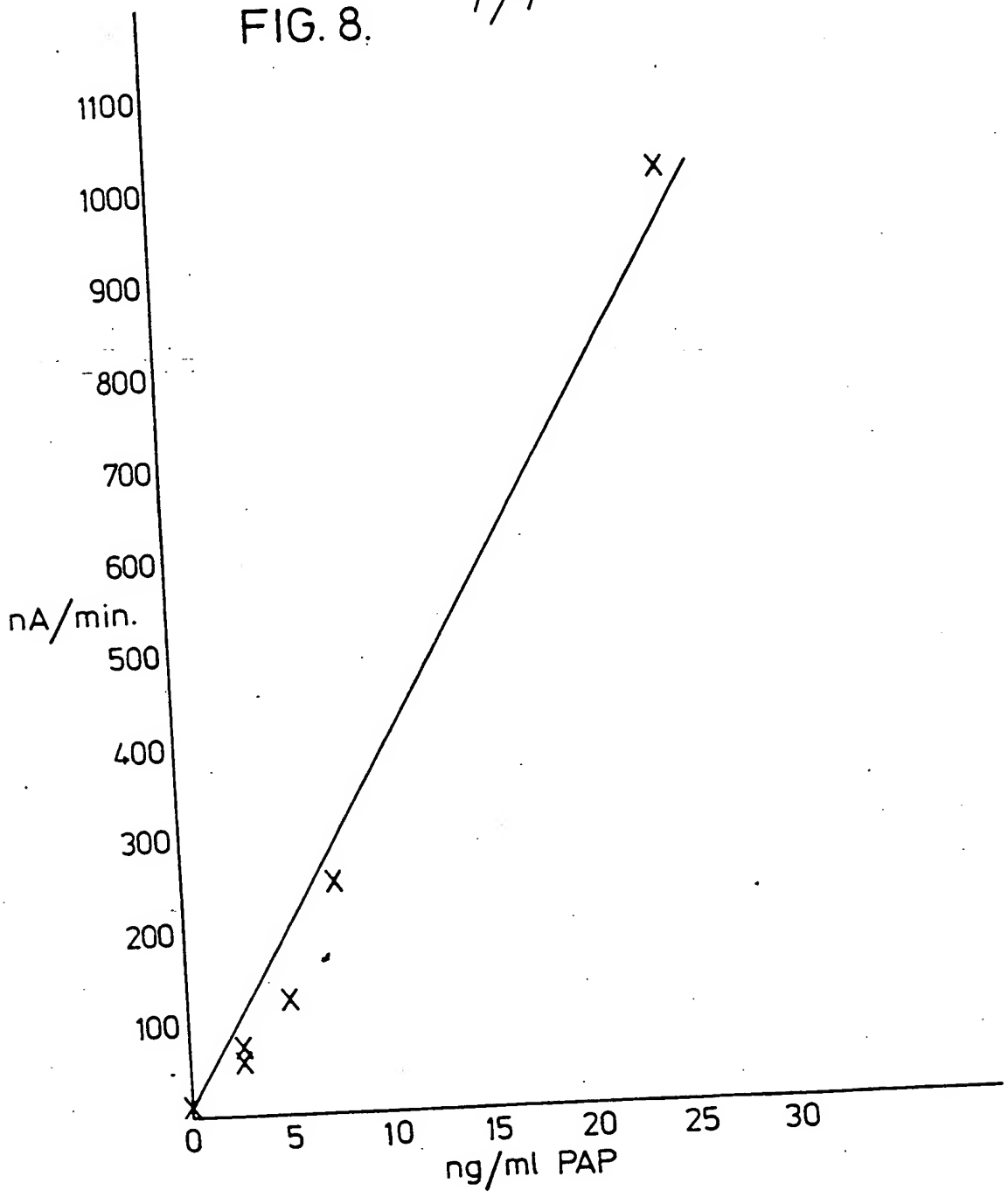
ng/ml PAP

FIG. 8. 7/7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1000
900
800
700
600
nA/min.
500
400
300
200
100
0
5
10
15
20
25
30
ng/ml PAP

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00590

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 33/53; C 12 M 1/40

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
IPC[4]	G 01 N; C 12 M

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with Indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,Y	EP, A, 0142301 (SERONO DIAGNOSTICS LIMITED) 22 May 1985 see the entire document	1,5,9,11,19
A	--	10
Y	EP, A, 0060123 (C.H. SELF) 15 September 1982 see the entire document (cited in the application)	1-5,7,9,11, 16,19,20
A	--	6,8
Y	Analytical Chemistry, vol. 52, no. 11, September 1980 (Easton, Pennsylvania, US) W.J. Blaedel et al.: "Reagentless enzyme electrodes for ethanol, lactate and malate", see pages 1691-1697	1-5,7,9,11, 16,19,20
A	-- US, A, 4156180 (P.A. ANNEN et al.) 22 May 1979 see claims 1,10-12,19,37	12-15
A	-- US, A, 3984766 (D.D. THORNTON) 5 October	./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th March 1986	21 AVR. 1986
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	1976 see the abstract and claims 1-5,14 --	12-15
A	Biotechnology and Bioengineering, vol. 25, no. 3, March 1983 (New York, US) J.P. Kernevez et al.: "Determination of substrate concentrations by a computerized enzyme electrode", see pages 845,852-854 --	14,15
A	US, A, 4334880 (M.K. MALMROS) 15 June 1982 ------	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 85/00590 (SA 11719)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/04/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		JP-A-	60127450	08/07/85
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		FR-A,B	2395509	19/01/79
		DE-A-	2827313	18/01/79
		JP-A-	54037878	20/03/79
		CA-A-	1106445	04/08/81
		SE-A-	7806236	25/12/78
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		DE-A-	2546632	29/04/76
		FR-A,B	2331033	03/06/77
		GB-A-	1529715	25/10/78
		CA-A-	1039809	03/10/78
		JP-A-	52001083	06/01/77
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